

EXHIBIT D

Hypertension Induced by Aortic Coarctation Above the Renal Arteries Is Associated With Immune Cell Infiltration of the Kidneys

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Background: Renal tubulointerstitial infiltration of activated T cells and macrophages is invariably present and plays a role in elevation of arterial pressure in nearly all animal models of hypertension (HTN). The role, if any, of elevated renal arterial pressure in the pathogenesis of this inflammatory process is uncertain. Also unclear is whether the cellular infiltration is caused by the local activation of immune cells in the kidney or a consequence of leukocyte activation in the systemic circulation.

Methods: We studied activation of peripheral blood leukocytes and cellular infiltration in the kidneys of Sprague-Dawley rats with abdominal aorta coarctation (banding) above renal arteries, which causes severe HTN proximal but not distal to coarctation.

Results: Compared with the sham operated controls, the aorta-banded group exhibited tubulointerstitial accumulation of activated T cells, macrophages, angiotensin-II positive cells, leukocyte function-associated antigen-1 integrin expressing cells, increased nitrotyrosine abundance

(a measure of oxidative stress), and increased macrophage chemoattractant protein-1 in the kidneys which are not exposed to HTN in this model. These findings were associated with the activation of the circulating leukocytes in the aorta-banded animals.

Conclusions: Increased baromechanical stress is not a requisite for accumulation of T cells and macrophages in the kidney in the coarctation-induced HTN and possibly in other hypertensive disorders. On the contrary, renal hypoperfusion and the consequent activation of renin-angiotensin system may mediate this process by promoting local induction of chemoattractant and inflammatory cytokines. The observed tubulointerstitial inflammation in this model is associated with leukocyte activation in the systemic circulation. *Am J Hypertens* 2005;18:1449–1456
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Key Words: Inflammation, hypertension, integrin expression, lymphocytes, macrophages.

Renal infiltration of activated T cells and macrophages is a feature common to most, if not all, experimental and genetic rat models of hypertension. This phenomenon has been shown to play a role in the initiation and maintenance of hypertension induced by nitric oxide synthase inhibition, angiotensin II infusion, and protein overload proteinuria, as well as that seen in spontaneously hypertensive rats and in double transgenic rats for the human renin and angiotensinogen genes.^{1–6} This causal association is supported by the observations that the reduction of renal tubulointerstitial infiltration by either immunosuppressive treatment or inhibition of the proinflammatory transcription factor, nuclear factor- κ B (NF κ B), can ameliorate hypertension.^{1–7} It is of note that

evidence of immune cell activation in hypertensive models is not limited to renal tubulointerstitial inflammation. In fact, several studies have provided compelling evidence for activation and degranulation of circulating leukocytes in spontaneously hypertensive rats,⁸ salt-sensitive Dahl rats,⁹ genetically normotensive rats made hypertensive,¹⁰ and human beings with essential hypertension.¹¹

Taken together the observations cited above point to the association of hypertension with both renal and systemic inflammation. However the role, if any, of elevated renal arterial pressure in the pathogenesis of the interstitial inflammatory process of the kidney remains uncertain. Moreover it is not clear whether this cellular infiltration is caused by events resulting from pressure-induced shear

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stress in the renal circulation or whether it is a consequence of systemic events involving circulating leukocytes.

In an attempt to address these issues we studied rats with abdominal aorta coarctation above the renal arteries, which causes severe hypertension in the arterial tree proximal but not distal to the stenosis. The results showed significant tubulointerstitial accumulation of activated T cells and macrophages in the kidneys of the study animals when compared with the sham operated controls. Because the kidneys in this model do not reside in the hypertensive zone of the body, the data indicate that tubulointerstitial infiltration of immune cells may be driven by factors other than elevated renal arterial pressure. The findings of concomitant activation of circulating leukocytes and increased macrophage chemoattractant protein-1 (MCP-1) expression in the kidney suggest that renal infiltration of immune cells may be a consequence of their activation in the systemic circulation in association with local chemoattractant cytokine expression.

Methods

Experimental Animals

The rats used in the present study were male Sprague-Dawley rats purchased from Taconic Biotech (Germantown, NY) with an average weight of 150 g with abdominal aorta coarctation above the renal arteries and sham operated control rats ($n = 6/\text{group}$). The surgical procedure had been carried out under general anesthesia with sodium pentobarbital at the Taconic Biotech facilities as described below. The animals were housed in climate-controlled, light-regulated institutional facilities with 12-h light and dark cycles and received regular rat feed and free access to water. After 3 weeks all animals were killed by exsanguination under general anesthesia (50 mg Nembutal [Abbot Laboratories, Chicago, Ill] intraperitoneally) by cardiac puncture between 8 AM and 11 AM. Kidneys were harvested, and coronal sections were fixed in formalin and used for histologic and immunohistologic studies.

Studies were approved by the Animal Care Institutional Committee of the University of California Irvine.

Surgical Procedures

A 2-cm dorsal midline incision was made with its cranial terminus at the level of the 13th rib. The left abdominal wall was opened 1.5 cm lateral to the spine. The abdominal aorta was located and the right renal artery was identified to ensure that constriction is applied cranial to both renal arteries. Curved forceps were inserted behind the aorta and a surgical silk was threaded behind the aorta. A blunt needle (23 G, outside diameter 0.65 mm), was then placed over the aorta and the silk tied snugly around the aorta and needle. The needle was then slid out from under the knot, leaving the aorta constricted to the external diameter of the needle. The abdominal wall was closed

with silk suture and the skin incision was closed with wound clips. Animals were closely monitored postoperatively for signs of decreased blood flow to the hind limbs. The sham procedure was performed identically without banding the aorta.

Blood Pressure and Renal Function

In a separate group of similarly operated (banded and sham) animals, blood pressure (BP) was directly measured by a carotic cannula as described earlier.¹² Both BP and renal function in the banded and sham operated rats were measured at the end of the experiment and have previously been reported.^{13,14}

Histologic and Immunohistologic Studies

All histologic and immunohistologic studies were done in a blinded fashion. Light histology was evaluated in sections stained with hematoxylin and eosin and periodic acid-Schiff stainings. Glomerular and tubulointerstitial changes were evaluated as detailed in previous communications.¹⁻⁴

Immunoperoxidase methodology was used to determine macrophage (ED1 positive cells) and lymphocyte (CD5 positive cells) infiltration, leukocyte function-associated antigen-1 (LFA-1) angiotensin II positive cells, and MCP-1 in the kidney as described in previous work.¹⁻⁴

Positive cells detected with the immune stainings were evaluated separately in the glomeruli (positive cells/glomerular cross section (gcs)) and in tubulointerstitial areas (positive cells/mm²).¹⁻⁴ Expression of MCP-1 was evaluated by computer-assisted image analysis done in the entire cortical region of the biopsy samples. Computer-assisted image was done with and Olympus BX51 System Microscope and DP70 microscope Digital camera, with Sigma Pro (Leesburgh, VA) image analysis software.

Antisera

Antisera used were the following: Anti-CD5 and anti-ED1 monoclonal antibodies (Biosource, Camarillo, CA) were used to identify lymphocytes and macrophages, respectively. Anti-LFA-1 antibody (anti-CD18, Seikegaku Corp., Tokyo, Japan). Rabbit antihuman angiotensin II antiserum with cross-reactivity to rat angiotensin II (Peninsula Laboratories, San Carlos, CA) was used to identify angiotensin II positive cells as detailed in previous communications. Specificity of the staining was tested by preincubating the antibody with human angiotensin II as detailed elsewhere.¹⁵ Rabbit polyclonal antirat MCP-1 was purchased from Biosource International (Camarillo, CA). Secondary biotin-conjugated affinity-pure antibodies with minimal reactivity to rat serum proteins were purchased from Accurate Chemical and Scientific Co. (Westbury, NY). Non-relevant antibodies were used for negative control studies.

Assays for the Generation of Hydrogen Peroxide

Production of reactive oxygen species in peripheral blood leukocytes was assessed by flow cytometry using fluorescent probes and a FACSort System (Becton-Dickinson, San Jose, CA). Whole blood was drawn by cardiac puncture and 100 μ L of blood was incubated with 0.125 μ g/mL of dihydrorhodamine-123 (DHR, for measurement of hydrogen peroxide) for 15 min at 37°C. The blood cells incubated without DHR were used as controls. Cells were precipitated by centrifugation and supernatant was discarded. The cell pellets were resuspended in 50 μ L of phosphate buffered saline (PBS) and stained with different blood cell marker antibodies. For analysis of CD4+ or CD8+ T lymphocytes blood cells were incubated for 30 min at room temperature with PE-conjugated anti-CD4 or anti-CD8 mAb. For analysis of monocytes or granulocytes, cells were incubated with biotin-conjugated HIS 48 mAb (specific for monocyte and granulocyte) for 30 min at room temperature. After being washed twice with PBS, cells were incubated with PE-conjugated streptavidin. The red blood cells were then lysed by incubation (for 10 min at room temperature) with 2 mL of flow cytometry (FACS) lysing solution. The leukocytes were washed twice with PBS, then resuspended and fixed in 0.5 mL of 1 % paraformaldehyde. The suspension of fixed cells was used for flow cytometry.

Flow Cytometry

Flow cytometry was carried out by dual-color analysis using a FACSort flow cytometer and CellQuest software (Becton-Dickinson, San Jose, CA). The mean leukocyte count was 3,200/mm³ in controls and 3,900/mm³ in the banded group. For each sample, data from 30,000 cells were collected and displayed in dot plot of forward scatter and side scatter. The cells were gated for lymphocytes, monocytes, or polymorphonuclear cells. The CD4+ or CD8+ T lymphocytes were subsequently gated in dot plot of side scatter and FL2 (PE-conjugated mAb) channel. Monocytes were further gated for HIS48+ cells in dot plot of side scatter and FL2 (PE-conjugated streptavidin) channel. Mean fluorescence intensity in the gated cell population was measured in FL1 channel for DHR. Data were presented as mean fluorescence intensity after background fluorescence intensity of controls was subtracted.

Integrins and T Cell Activation

Expression of integrin on the surface of peripheral blood leukocytes was assessed by flow cytometry using FITC-conjugated anti-CD11a (α L subunit of integrin LFA-1) mAb, anti-CD11b (α M subunit of integrin CR3) mAb, and anti-CD18 (integrin β 2 subunit) mAb. To assess T cell activation, cell surface expression of IL-2 receptor was measured by flow cytometry using an anti-CD25 (IL-2 receptor α chain) mAb. Briefly, 100 μ L of peripheral blood were incubated with each of the above antibodies

along with PE-conjugated anti-CD4 or anti-CD8 mAb for 30 min at room temperature. The cells were then incubated in FACS lysing solution for 10 min and washed twice with PBS to remove red blood cells. Leukocytes were collected by centrifugation, resuspended, and fixed in 0.5 mL of 1% paraformaldehyde before being used for flow cytometry. Likewise splenic cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-CD11a, anti-CD11b, anti-CD18, or anti-CD25 mAb along with PE-conjugated anti-CD4 or anti-CD8 mAb for 30 min at room temperature. Cells were washed twice with PBS, resuspended in 1% paraformaldehyde, and used for flow cytometry. Cells incubated with FITC-conjugated mouse IgG were used as isotype control.

Nitrotyrosine Abundance

Kidneys were processed for determination of nitrotyrosine as detailed in a previous communication.¹² Briefly, tissues (25% wt/vol) were homogenized using a polytron homogenizer in a solution containing 50 mmol/L tris-HCl (pH7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EGTA, aprotinin, leupeptin, and pepstatin (1 μ g/mL each), 1 mmol/L Na₃VO₄, and 1 mmol/L NaF at 0° to 4°C. Homogenates were centrifuged at 12,000 *g* for 5 min at 4°C, and the supernatants were used for nitrotyrosine abundance by Western blot methodology with antinitrotyrosine monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY) as described before.¹³ Protein determination was done using a commercially available BCA protein assay kit (Pierce Inc., Rockford, IL).

Statistical Analysis

Comparison of data obtained in banded and sham operated rats was done with unpaired Student *t* tests. Comparisons between multiple groups were done with analysis of variance and when *P* values were < .05, Tukey post-tests were used to identify groups among which there were significant differences. Data are presented as mean \pm standard error of the mean. *P* values < .05 were considered to be significant.

Results

General Data

As previously reported,^{12,13} the abdominal aortic banding induced significant (*P* < .01) increments in systolic BP (203 \pm 9 mm Hg *v* 137 \pm 1), mean arterial pressure (162 \pm 4 mm Hg *v* 115 \pm 2), and pulse pressure (74 \pm 7 mm Hg *v* 42 \pm 1). As noted in previous communications,^{12,13} creatinine clearance (in milliliters per minute) was comparable in the banded rats (2.3 \pm 0.5) and the sham operated controls (2.5 \pm 0.9), as was the urinary protein excretion (mg/24 h) in banded (15 \pm 2) and sham operated control rats (14 \pm 1).

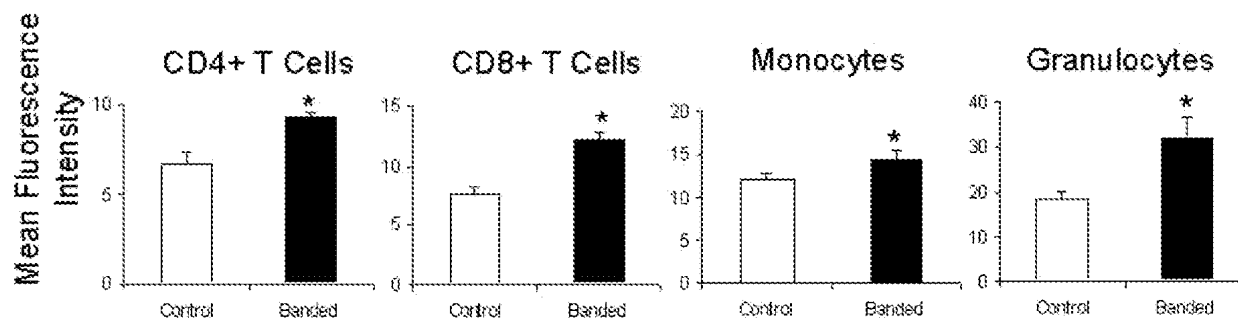


FIG. 1. Hydrogen peroxide production by CD4+ and CD8+ T lymphocytes, monocytes, and granulocytes in the blood of rats with abdominal aorta banding-induced hypertension (**filled columns**) and the sham operated control rats (**open columns**). $n = 6/\text{group}$; $*P < .05$.

Light Microscopy

No significant histologic abnormalities were found by light microscopy in the kidneys of the banded animals. Renal biopsy samples were essentially normal and were similar in the banded and sham operated groups.

Hydrogen Peroxide Production by Peripheral Blood Leukocytes

The production of hydrogen peroxide by CD4+ and CD8+ T cells, monocytes, and granulocytes, as determined by fluorescent probes, was significantly increased in the banded group¹⁰ (Fig. 1).

Leukocyte Integrin Expression

The banded animals exhibited a significant upregulation of CD18 on T helper cells. Mean values of CD11a and CD11b expression were also higher in the T helper cells but their increase did not reach statistical significance. Aortic banding did not increase integrin expression on T suppressor (CD8-positive) cells (Fig. 2).

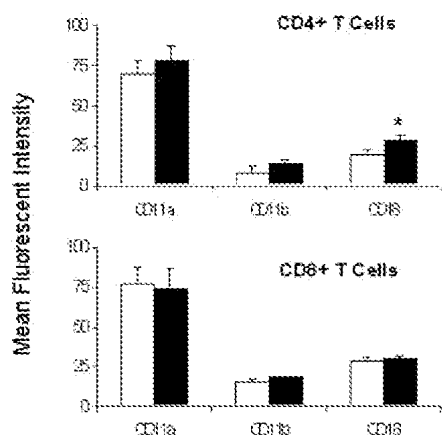


FIG. 2. Integrin expression in CD4 and CD8 T lymphocytes. The banded animals (**filled columns**) exhibited a significant upregulation in CD18 in T helper cells with respect to the sham operated normotensive animals (**open columns**). $n = 6/\text{group}$. $*P < .05$.

Infiltrating Lymphocytes and Macrophages in the Kidney

There were no significant differences in the lymphocytes infiltrating the glomerular tuft in the banded (0.30 ± 0.12 CD5+ cells/gcs) and control (0.33 ± 0.17) groups. Glomerular macrophages were also similar in the experimental and control groups (0.20 ± 0.12 ED1+ cells/gcs v. 0.17 ± 0.10). In contrast, there were highly significant ($P < .001$) increments in the cellular infiltration in the tubulointerstitial regions of banded animals that showed two- to threefold higher numbers of CD5+ (Fig. 3) and ED1+ cells/mm² (Fig. 4) than those found in the control group.

Integrin expressing infiltrating leukocytes (LFA-1 pos-

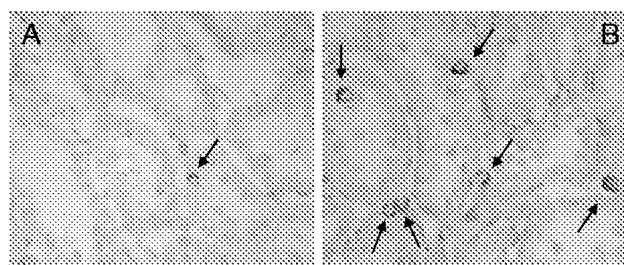
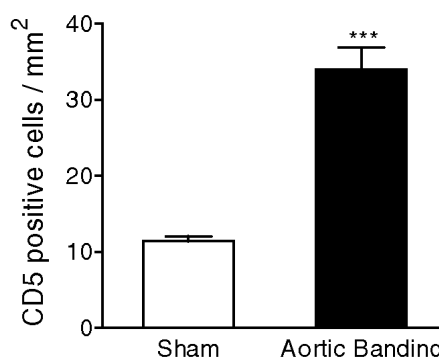


FIG. 3. Lymphocyte infiltration (CD5 positive cells) in tubulointerstitial areas of the kidney is induced by aortic banding above the renal arteries. $***P < .001$. Representative microphotographs of renal biopsy samples in sham operated (**A**) and banded (**B**) animals (anti-CD5 immunoperoxidase staining. Positive cells are shown with **arrows**. Original magnification $\times 400$).

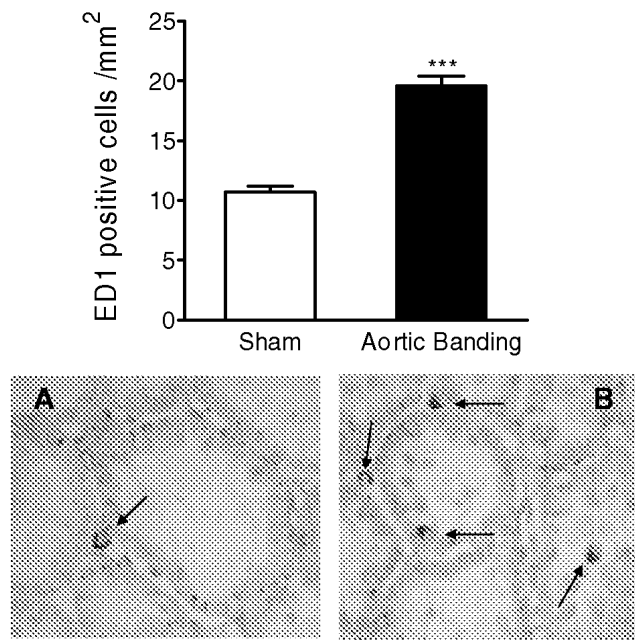


FIG. 4. Macrophage infiltration (ED1 positive cells) in tubulointerstitial areas of the kidney is induced by aortic banding above the renal arteries. *** $P < .001$. Representative microphotographs of renal biopsy samples in sham operated (**A**) and banded (**B**) animals (anti-ED1 immunoperoxidase staining. Positive cells are shown with arrows. (Original magnification $\times 400$).

itive cells) were more numerous in the glomeruli and tubulointerstitial areas of the renal biopsy samples of the rats with aortic banding compared with the sham operated controls (Fig. 5).

Angiotensin II Positive Cells

Angiotensin II positive cells were practically absent in the glomeruli in the banded and control rats. However in the tubulointerstitial areas of the banded rats the number of angiotensin II positive cells were 20-fold more numerous than in the sham operated rats (Fig. 6).

Nitrotyrosine Abundance

As shown in Fig. 7, nitrotyrosine content was significantly elevated in the aortic banded group of rats.

Expression of MCP-1

The expression of MCP-1 was increased sixfold ($P < .001$) in the tubulointerstitium of the group with aortic banding with respect to the values in the sham operated group (Fig. 8).

Discussion

There is a well established inter-relationship among hypertension, renal inflammation, and oxidative stress.^{16,17} It is also well known that pressure-related shear stress may trigger local inflammatory reactivity and oxidative

stress.^{18–20} However the role of baromechanical factors in the development of intrarenal inflammation is undefined, as local activation and attraction of immune cells to the kidney may be part of the systemic effects involving circulating leukocytes. Most experimental models of hypertension are not well suited to separate the effects of hemodynamic from those of circulating humoral factors. One exception is the hypertension induced by aortic banding above the renal arteries which results in increased BP in the upper body²¹ and renal hypoperfusion and stimulation of the renin-angiotensin system.^{22,23} In this model, upregulation of endothelial nitric oxide synthase (e-NOS) and endothelial dysfunction¹² increased nitrotyrosine abundance (a measure of reactive oxygen species interaction with nitric oxide)¹³ and upregulation of NADPH oxidase subunits,²⁴ have been found in the aorta segment above but not below the site of banding. These findings point to a baromechanically induced etiology, because circulating humoral factors are similar above and below the coarctation. In contrast the present studies demonstrate increased renal nitrotyrosine abundance and, because the kidneys are located below the aortic coarctation, their increased oxidative stress is not related to baromechanical factors. Reduced renal perfusion resulting from banding, in association with interstitial inflammation and increased intrarenal angiotensin II activity (see later here), likely

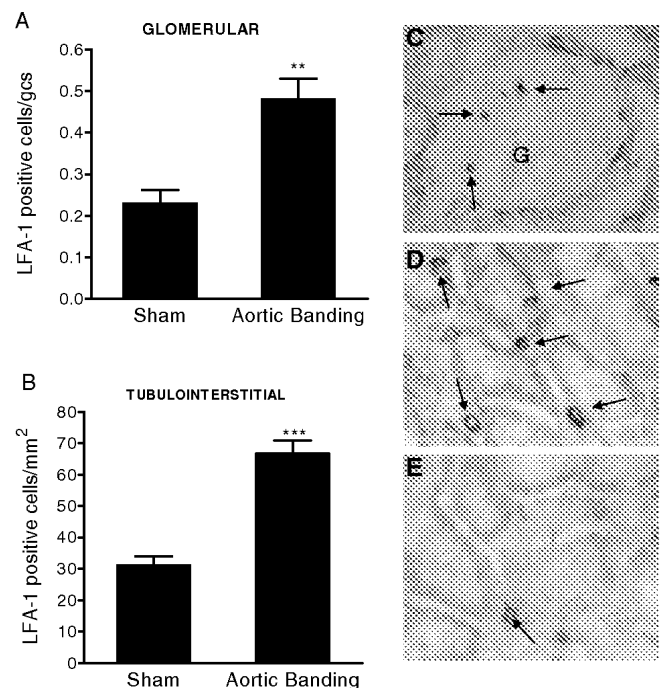


FIG. 5. Infiltration of leukocyte function-associated antigen-1 (LFA-1) positive cells (arrows) in glomeruli (**A**, glomeruli indicated as **G**) and tubulointerstitial areas (**B**) is increased in renal biopsy samples of rats with aortic coarctation (banding) above the renal arteries. ** $P < .01$, *** $P < .0001$). Representative microphotographs showing LFA-1 positive cells in biopsy samples of glomeruli (**B**) and tubulointerstitium (**C**) of banded animals. Sham operated animals had only occasional LFA-1 cells in tubulointerstitial areas (**D**) (immunoperoxidase staining, original magnification $\times 40$).

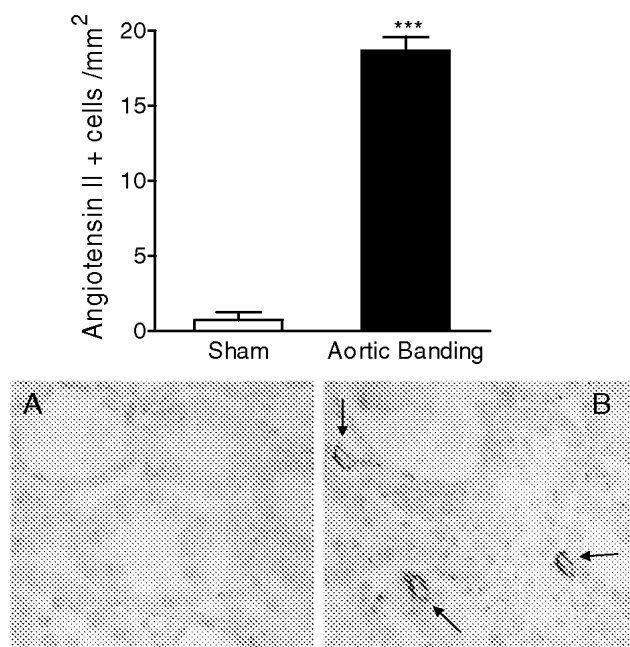


FIG. 6. Angiotensin II positive cells are increased in tubulointerstitial areas of biopsy samples obtained from rats with aortic banding above the renal arteries. *** $P < .001$. Representative microphotographs of biopsy samples in sham operated (**A**) and banded (**B**) rats (immunoperoxidase staining; original magnification $\times 40$).

combine to increase intrarenal oxidative stress in this model.

Tubulointerstitial infiltration of lymphocytes and macrophages, proliferative activity, and collagen deposition have been previously found to be present in the unclipped kidney in two-kidney, one-clip Goldblatt hypertension,^{25,26} whereas the clipped kidney was assumed to be protected from hypertension. If this were the case, then only baromechanical factors would be

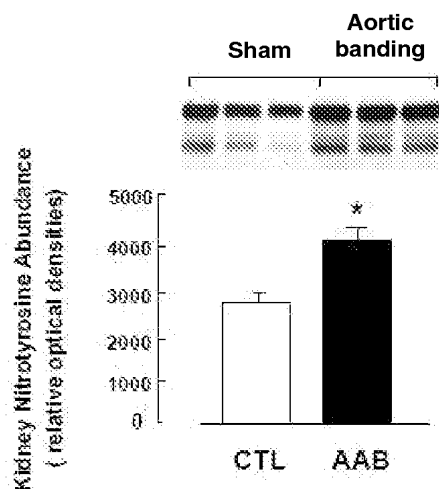


FIG. 7. Nitrotyrosine abundance is increased in the group of rats with aortic banding. Technical details are described in Methods. * $P < .05$.

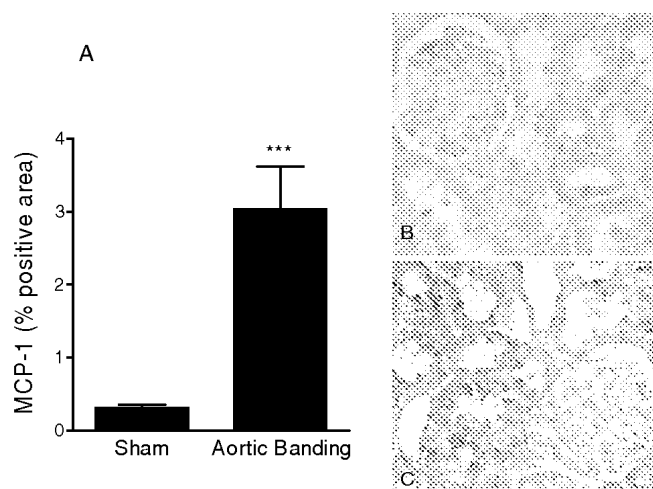


FIG. 8. (A) Macrophage chemoattractant protein-1 (MCP-1) is overexpressed as a result of aortic banding. *** $P < .001$. Microphotographs show the contrast observed in sham operated rats (**B**) and rats with aortic banding (**C**). Areas positive for MCP-1 are shown as dark brown staining in most epithelial tubular cells (immunoperoxidase staining; original magnification $\times 400$).

responsible for the tubulointerstitial immune infiltration of the kidneys. However, in the two-kidney, one-clip Goldblatt model several humoral factors can potentially induce immune cell infiltration in the kidney. Notable among them is systemic oxidative stress, as well as increased systemic and intrarenal angiotensin II activity. The latter is known to elicit immune cell infiltration as a manner observed with systemic angiotensin II infusions.^{27,28} The present study shows that in fact, infiltration of lymphocytes and macrophages and increased numbers of angiotensin II positive cells are present in the kidneys below the aortic coarctation. The observed increase in the number of angiotensin II positive cells in the banded animals points to heightened intra-renal angiotensin activity, which could potentially contribute to the characteristic reduction of sodium excretion in the underperfused kidney. Angiotensin II is known to induce proinflammatory cytokines²⁹ and specifically MCP-1,³⁰ as well as NAD(P)H oxidase-catalyzed superoxide generation.³¹ Therefore increased angiotensin II production in response to renal hypoperfusion could be responsible in part for both the local overexpression of MCP-1 and oxidative stress (elevated renal nitrotyrosine content) in the kidneys of the banded animals. Consequently renal hypoperfusion, as opposed to increased renal arterial pressure, is involved in this instance.

Heterodimerization of CD18 with CD11a on the cell membrane leads to formation of the LFA-1 integrin. The substance LFA-1 serves as a cell surface receptor that mediates the interaction of lymphocytes with endothelial cells and other leukocyte via binding to the intercellular adhesion molecules on the surface of these cells. Accordingly LFA-1 plays an important role in the inflammatory response as well as cell adhesion to endothelial surface and tissue infiltration.³² The

upregulation of the CD18 shown in the T helper population in the present studies (Fig. 2) suggest that increased adhesion, interstitial migration, and activation of circulating lymphocytes may play a role in the interstitial accumulation of immune cells in conditions associated with increased BP. In fact increased numbers of LFA-1 positive cells were found in the kidneys of banded animals (Fig. 5). The total number of LFA-1 positive cells found in the kidneys of the banded animals approximates the combined number of lymphocytes and macrophages infiltrating the kidney, which suggests that the vast majority of infiltrating immunocompetent cells are activated.

During our relatively limited observation period the kidneys did not show light microscopic changes, but is possible that in long term studies structural alterations may develop. However the chronic damage in the underperfused kidneys is likely to be less than that observed in the unclipped side of the two-kidney, one-clip Goldblatt model, because in the latter instance the baromechanical stress and humoral factors act in concert to induce inflammation and collagen deposition in the kidney.

In summary our studies show that aortic coarctation above the kidneys is associated with tubulointerstitial infiltration of immune cells. Therefore increased pressure in the renal circulation is not a requisite for the accumulation of lymphocytes and macrophages in the kidney in this model. On the contrary renal hypoperfusion and the consequent activation of renin-angiotensin system may mediate this process via local induction of chemoattractant and inflammatory cytokines. The observed tubulointerstitial inflammation in this model is associated with, and perhaps partly caused by, activation of leukocytes in the systemic circulation.

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